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The Mode of Action of Chicory Roots on Skatole Production in Entire Male Pigs Is neither via Reducing the Population of Skatole-Producing Bacteria nor via Increased Butyrate Production in the Hindgut

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ABSTRACT The effect of high levels of dietary chicory roots (25%) and intracecal exogenous butyrate infusion on skatole formation and gut microbiota was investigated in order to clarify the mechanisms underlying the known reducing effect of chicory roots on skatole production in entire male pigs. A Latin square design with 3 treatments (control, chicory, and butyrate), 3 periods, and 6 animals was carried out. Chicory roots showed the lowest numerical levels of skatole in both feces and plasma and butyrate infusion the highest. In the chicory group, an increased abundance of the skatole-producing bacterium *Olsenella scatoligenes* compared to the control group ($P = 0.06$), and a numerically higher relative abundance of *Olsenella* than for the control and butyrate groups, was observed. Regarding butyrate-producing bacteria, the chicory group had lower abundance of *Roseburia* but a numerically higher abundance of *Megasphaera* than the control group. Lower species richness was found in the chicory group than in the butyrate group. Moreover, beta diversity revealed that the chicory group formed a distinct cluster, whereas the control and butyrate groups clustered more closely to each other. The current data indicated that the skatole-reducing effect of chicory roots is neither via inhibition of cell apoptosis by butyrate nor via suppression of skatole-producing bacteria in the pig hindgut. Thus, the mode of action is most likely through increased microbial activity with a corresponding high incorporation of amino acids into bacterial biomass, and thereby suppressed conversion of tryptophan into skatole, as indicated in the literature.

IMPORTANCE Castration is practiced to avoid the development of boar taint, which negatively affects the taste and odor of pork, and undesirable aggressive behavior. Due to animal welfare issues, alternatives to surgical castration are sought, though. Boar taint is a result of high concentrations of skatole and androstenone in back fat. Skatole is produced by microbial fermentation in the large intestine, and therefore, its production can be influenced by manipulation of the microbiota. Highly fermentable dietary fiber reduces skatole production. However, various theories have been proposed to explain the mode of action. In order to search for other alternatives, more efficient or less expensive, to reduce skatole via feeding, it is important to elucidate the mechanism behind the observed effect of highly fermentable dietary fiber on skatole. Our results indicate that highly fermentable dietary fiber does not affect skatole production by reducing the number of skatole-producing bacteria or stimulating butyrate production in the large intestine.

KEYWORDS boar taint, butyric acid, chicory roots, gut microbiota, pigs, skatole

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Boar taint is an offensive odor and flavor released upon heating meat from some pubertal or sexually mature entire male pigs, making the meat undesirable for human consumption, with a consequent negative economic impact for the pig industry (1). Skatole (3-methylindole), which has a fecal-like odor, is produced by microbial degradation of L-tryptophan (TRP) in the hindgut of pigs and is, together with androstenone, the main compound responsible for boar taint (2). Skatole production is limited to a few bacterial species, of which only one, *Olsenella scatoligenes*, has been isolated from the pig gut so far (3). Skatole is produced in the hindgut of both pig sexes; however, higher concentrations are measured in the back fat of entire male pigs, probably due to reduced skatole metabolism in the liver compared to that in females (4, 5).

Surgical castration of male pigs within the first week of life is a common practice in many countries to prevent boar taint. However, due to welfare concerns, the European Union aimed at ending castration of pigs by January 2018 (Declaration of Brussels, 2010), turning boar taint into a challenge in pig production. Although androstenone also contributes to boar taint, the significant contribution of skatole to the meat odor perception indicates that reducing the skatole concentration may be an effective means to increase consumer acceptance (6). Adding certain fermentable dietary fiber (DF) sources to the feed prevents boar taint by reducing skatole production, making this strategy an alternative to surgical castration (7). Dietary fiber sources rich in inulin, such as chicory roots and Jerusalem artichoke, have been shown to be the most effective fiber sources (1).

Three hypotheses have been proposed to explain the impact of fermentable DF on skatole production in the hindgut of pigs: (i) supplementation of fermentable DF leads to an increased butyrate concentration in the colon, which reduces cell apoptosis, thereby reducing the availability of TRP for skatole formation from gut mucosa cell debris (8); (ii) high levels of fermentable fiber in the hindgut, due to its high levels in the feed, increase the microbial activity, resulting in a higher incorporation of amino acids in bacterial biomass and a corresponding suppression of conversion of TRP into skatole (9); and (iii) the effect of DF is elicited by modulating the composition of the microbiota toward one characterized by a decreased abundance of skatole-producing bacteria.

The impact of chicory roots and other highly fermentable fiber sources containing inulin on microbiota composition has been shown to vary, though. Diets containing chicory roots have been reported to result in increased colonic abundance of *Megasphaera elsdenii* (10), a dominant butyrate producer in pigs (11). Accordingly, it was found that feeding a diet with chicory roots and sweet lupins to growing pigs stimulated *Bifidobacterium thermoacidophilum* and *M. elsdenii* (12). Further, a Jerusalem artichoke-rich diet resulted in a reduced level of *Clostridium perfringens* in both the colon and rectum and a tendency toward decreased levels of *Enterobacteriaceae* in the colon (13). Further, inconsistent effects of inulin on pig gut microbiota have been observed (14). Selective enhancement of beneficial *Bifidobacterium* and *Lactobacillus* species was reported after the dietary inclusion of 4% (15–17) inulin, whereas others did not demonstrate such an effect when adding 2% (18) and 3% (19, 20) inulin. These reported results do not indicate a clear relationship between fibers shown to reduce skatole production and their impact on specific members of the microbiota. Regarding butyrate, it is the major product from microbial fermentation of inulin (21). Tsukahara et al. (22) reported increased concentration of short-chain fatty acids (SCFA) and the concomitant increased butyrate production when feeding piglets with fructo-oligosaccharides (FOS).

The experiment outlined in the current study was designed to investigate the mechanisms behind the reducing effect of chicory roots on skatole production in entire male pigs. For that, we aimed to determine whether, in the absence of high levels of fermentable fiber (chicory roots), butyrate elicits a skatole-reducing effect, and whether chicory roots influence the population of skatole-producing bacteria in the pig hindgut and thereby decrease the skatole concentration in feces and plasma.

TABLE 1 Feces/feed ratio, dry matter, and concentrations of organic acids and chemical compounds in feces of pigs in the experimental treatment groups[a]

Item measured	Value for treatment			SEM	*P* value		
	Control	Chicory	Butyrate		Treatment[b]	Period	Treatment × period
Feces/feed (g/kg)	478.3	605.7	513.7	47.3	0.31	0.38	0.03
Feces DM (%)	22.6	20.4	24.6	1.1	0.12	0.28	0.03
Organic acids (mmol/kg of wet feces)							
Acetic acid	75.9	79.3	74.3	4.4	0.62	0.86	0.48
Propionic acid	33.9	37.2	33.4	3.5	0.57	0.98	0.96
Butyric acid	21.8	16.2	17.9	4.2	0.55	0.93	0.79
Valeric acid	4.9	5.9	5.9	1.2	0.84	0.71	0.98
BCFA[c]	4.9	4.7	7.8	0.8	0.10	0.31	0.02
Chemical compounds (mg/kg of wet feces)							
Tryptophan	7.4	5.9	1.5	3.3	0.32	0.30	0.89
Indole propionic acid	1.8	6.3	0.8	1.6	0.10	0.49	0.57
Indole acetic acid	3.0	1.0	0.4	1.3	0.44	0.61	0.71
Skatole	15.7 AB	3.8 A	40.8 B	6.5	0.03	0.29	0.32
Indole	16.8	17.1	15.8	4.7	0.98	0.39	0.09
p-Cresol	58.0	63.1	118.9	12.9	0.05	0.28	0.02

[a]Values are least square means ($n = 6$). Different letters indicate significant differences ($P < 0.05$).
[b]The pigs in the control group received a standard diet and cecal infusion of NaCl, the butyrate group received a standard diet and cecal infusion of butyrate, and the chicory group received a chicory diet and cecal infusion of NaCl.
[c]BCFA, branched-chain fatty acids (including isobutyric and isovaleric acids).

RESULTS

The initial body weight (BW) of the animals was 41.1 ± 1.8 kg, and at the end of the experimental period, the BW was 62.4 ± 4.5 kg. Feed intakes in the control, chicory, and butyrate groups were 1.77, 1.80, and 1.68 kg/day, respectively. During the whole study period, the pigs gained 0.86 (control), 0.85 (chicory), and 0.78 (butyrate) kg/day. During the first period, one pig in the butyrate group lost 0.9 kg. During the third period, one pig in the control and one in the chicory group had diarrhea and received antibiotic (dihydrostreptomycin sulfate and benzylpenicillin procaine) treatment for 3 days. These animals showed not to be outliers in the response parameters tested in the current study; therefore, they were kept in the data set.

Amount of feces excreted and fecal SCFA concentration. A significant effect of the interaction between treatment and period was observed for ratio of feces excreted to feed intake and fecal dry matter (DM) (Table 1). This could be ascribed to a low value of the ratio in the control in period 3 and a high value of DM in the chicory in period 2 (data not shown). No impact of treatment was found on the fecal SCFA concentration except branched-chain fatty acids (BCFA) (Table 1). A significant effect of the interaction between treatment and period was detected, probably due to a high BCFA concentration in the chicory in period 2. However, the overall tendency was that the BCFA (isobutyric and isovaleric acid) concentration was numerically higher in the butyrate group than in the other two groups ($P_{treatment} = 0.10$).

Indolic and phenolic compounds in feces. No significant impact of treatment was found on the fecal content of TRP, indole propionic acid (IPA), or indole acetic acid (IAA), but a tendency for IPA ($P_{treatment} = 0.10$) was observed, with the highest value measured in the chicory group (Table 1). A decrease in fecal skatole concentration was found in animals receiving chicory roots compared with that of animals from the butyrate group (3.8 versus 40.8 mg/kg; $P_{treatment} = 0.03$). A significant effect of the interaction between treatment and period was observed for *p*-cresol, which could be explained by a high level in the chicory group in period 2 (data not shown). However, the average fecal *p*-cresol concentration was highest in the butyrate group ($P_{treatment} = 0.05$).

Skatole and indole concentrations in plasma. Sampling time (from 30 min before feeding to 6 h after feeding) had no effect on the level of plasma skatole or indole (data

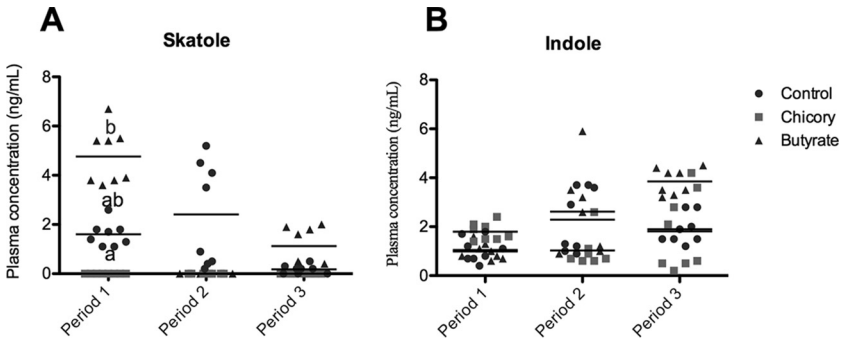

FIG 1 Plasma skatole (A) and indole (B) concentrations in the pigs from three experimental treatments and in the three experimental periods. Within each period, blood samples were taken from two pigs in each group at −30 min and at 2, 4, and 6 h. The control group received a standard diet and cecal infusion of NaCl, the butyrate group received a standard diet and cecal infusion of butyrate, and the chicory group received a chicory diet and cecal infusion of NaCl. The three lines in each period represent least square means (lsmeans) of each experimental treatment. Different letters indicate significant differences among treatments ($P < 0.05$).

not shown). In accordance with the fecal data presented in Table 1, average plasma skatole levels for the three periods were numerically lowest (0.0 ng/ml) in the chicory group and highest (2.0 ng/ml) in the butyrate group (Fig. 1A). However, due to high variation among pigs and a significant effect of period, a significant difference among treatments was observed only in the first period, in which a higher concentration of skatole in the butyrate group than in the chicory group was detected (4.7 versus 0.0 ng/ml; $P = 0.01$), with the same tendency when compared to the control group (4.7 versus 1.7 ng/ml; $P = 0.10$) (Fig. 1A). The plasma indole concentrations at the tested time points were not affected by either chicory root supplementation or butyrate infusion (Fig. 1B).

***Olsenella scatoligenes* and *Olsenella* abundance in feces.** Although the lowest numerical fecal and plasma skatole concentrations were found in the chicory group, an increase of *O. scatoligenes* numbers, quantified by quantitative real-time PCR (qPCR), was detected in this group compared to the control group (6.2 versus 4.7 $\log_{10}$ cells/g; $P = 0.06$) (Fig. 2A). Similar to *O. scatoligenes*, *Olsenella* abundance in feces was higher in the chicory group than in the control and butyrate groups (0.52% versus 0.06% and 0.05%, respectively), but no significant difference was achieved (Fig. 2B).

The overall fecal microbiota composition of entire male pigs. A total of 18 fecal samples were collected in the current study (1 per pig and treatment), and a total of 1,200,679 high-quality sequences with a minimum of 40,833 sequences per sample

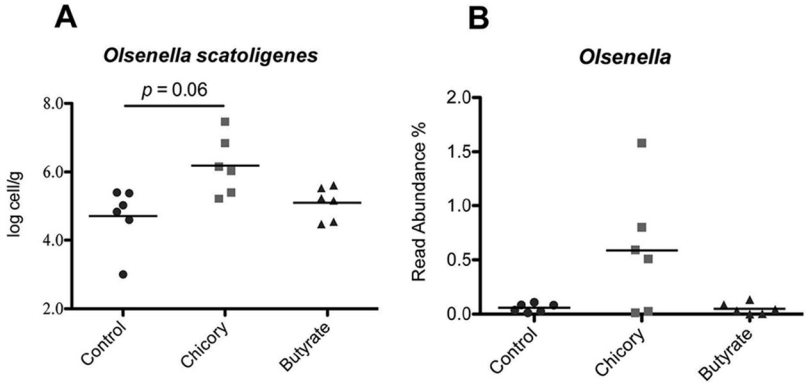

FIG 2 Fecal *O. scatoligenes* number, determined by quantitative real-time PCR and presented as $\log_{10}$ cell/g of wet feces (A), and *Olsenella* abundance, classified by RDP classifier and presented as read abundance in the pigs from three experimental treatments (B). The three lines in each period represent lsmeans for each treatment.

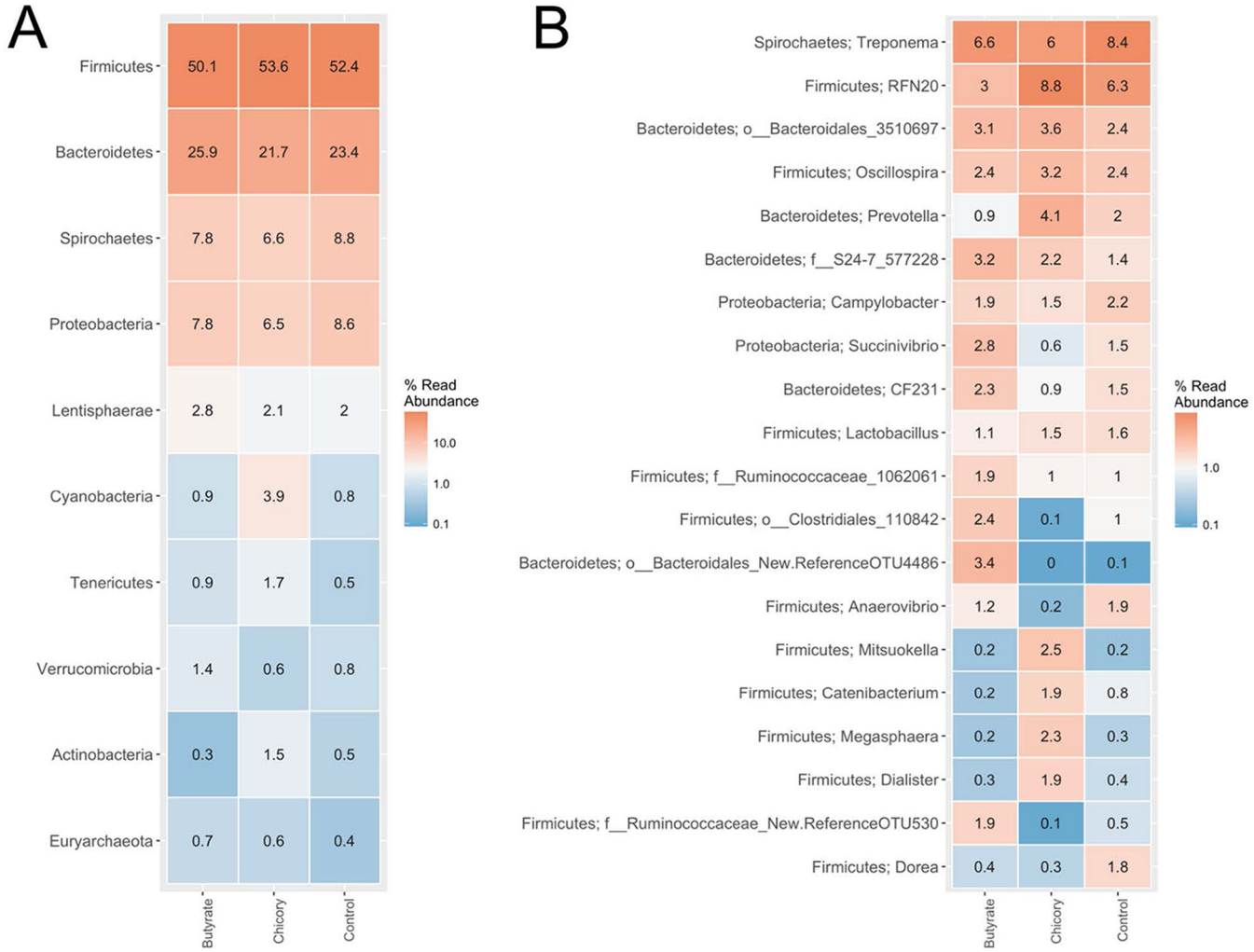

FIG 3 Overall fecal microbiota composition. (A) Relative abundances of the 10 most abundant phyla in the three treatments; (B) relative abundances of the 20 most abundant genera in the three treatments. The taxonomy is shown as the phylum and genus classification where they could be classified to a genus; otherwise they are shown as the phylum and OTU classification.

(mean = 66,772; read length = 303 to 556 bp) were obtained. After removal of low-abundance operational taxonomic units (OTUs; <0.005%), the data contained 994 OTUs, representing 17 phyla and 95 genera. The microbiota across all samples was dominated by the *Firmicutes* and *Bacteroidetes* phyla, accounting for 75.7% of total sequences (Fig. 3A), and the average ratio between *Firmicutes* and *Bacteroidetes* for all treatments was between 1.9 and 2.5. Other phyla were present at percentages lower than 10% (e.g., *Spirochaetes* [7.7%], *Proteobacteria* [7.6%], *Lentisphaerae* [2.3%], *Cyanobacteria* [1.9%], and *Tenericutes* [1.0%]). In general, the abundances of the various phyla in the different treatment groups were similar.

The classified bacterial genera detected at ≥1% average relative abundance were (in decreasing order) *Treponema*, RFN20, *Oscillospira*, *Prevotella*, *Campylobacter*, *Succinivibrio*, CF231, *Lactobacillus*, *Anaerovibrio*, *Mistuokella*, and *Catenibacterium* (Fig. 3B). In addition, the genera *Roseburia*, *Dorea*, *Bacteroides*, *Coprococcus*, and *Ruminococcus* also accounted for ≥1% of the relative abundance in the control pigs (data not shown).

Changes in the relative abundance of genera among the different treatments were compared by DESeq Wald test. A total of 6 genera (*Bacteroides*, *Collinsella*, and 4 unclassified) had different abundances ($P < 0.05$), and 10 genera (*Roseburia, Paludibacter*, *Enterococcus*, *Coprococcus*, and 6 unclassified) tended ($P \leq 0.10$) to be different among the different treatment groups (Table 2). *Bacteroides*, *Roseburia*, *Enterococcus*,

TABLE 2 Relative abundances of bacterial taxon groups in the fecal microbiota of pigs in the experimental treatments[a]

Taxonomy[b]	Relative abundance (%) in group			*P* value
	Control	Chicory	Butyrate	
Proteobacteria; o_GMD14H09_New.ReferenceOTU57	2.20	—	—	<0.01
Bacteroidetes; *Bacteroides*	1.45	—	—	0.01
Firmicutes; f_*Ruminococcaceae*_344523	0.11	0.02	—	0.02
Firmicutes; o_*Clostridiales*_187767	—	—	1.31	0.02
Cyanobacteria; o_YS2_New.ReferenceOTU1001	—	0.11	0.19	0.02
Actinobacteria; *Collinsella*	0.25	0.41	0.01	0.03
Bacteroidetes; f_S24-7_New.ReferenceOTU3921	0.40	—	—	0.05
Firmicutes; f_*Lachnospiraceae*_708680	1.77	0.31	0.16	0.05
Firmicutes; *Roseburia*	1.93	0.18	0.14	0.05
Firmicutes; o_*Clostridiales*_New.ReferenceOTU3458	—	—	0.41	0.06
Firmicutes; f_*Lachnospiraceae*_183362	—	—	0.11	0.07
Bacteroidetes; *Paludibacter*	—	—	0.09	0.08
Firmicutes; *Enterococcus*	0.16	—	—	0.08
Firmicutes; *Coprococcus*	0.87	0.61	0.18	0.10
Firmicutes; f_*Lachnospiraceae*_New.ReferenceOTU522	0.23	—	0.03	0.10
Bacteroidetes; o_*Bacteroidales*_New.ReferenceOTU4486	0.06	—	3.60	0.10

[a]Values are least square means ($n = 6$).
[b]Taxonomies are shown as a phylum and genus classification where they could be classified to a genus; otherwise, they are shown at phylum and OTU classification. Only genera significantly affected ($P \leq 0.05$) or with tendency to be affected ($P \leq 0.10$) by treatment are presented. —, trace abundance (<0.01%).

and *Coprococcus* were most abundant in the control group and *Collinsella* was most abundant in the chicory group, while *Paludibacter* was most abundant in the butyrate group. Six of the unclassified genera belonged to *Firmicutes*, two belonged to *Bacteriodetes*, and one each belonged to *Proteobacteria* and *Cyanobacteria*.

Alpha diversity. Samples were rarefied to 39,346 sequences to account for unequal numbers of sequences between samples before calculating alpha diversity indices (Fig. 4). The average of Chao 1 (a nonparametric estimator of OTU richness), the average number of observed OTUs (a pure estimator of community richness), and the average value for phylogenetic distance (PD) (the degree of phylogenetic divergence between sequences within each sample) were highest in the butyrate group and lowest in the chicory group ($P < 0.05$), whereas the control group had intermediate values. A similar

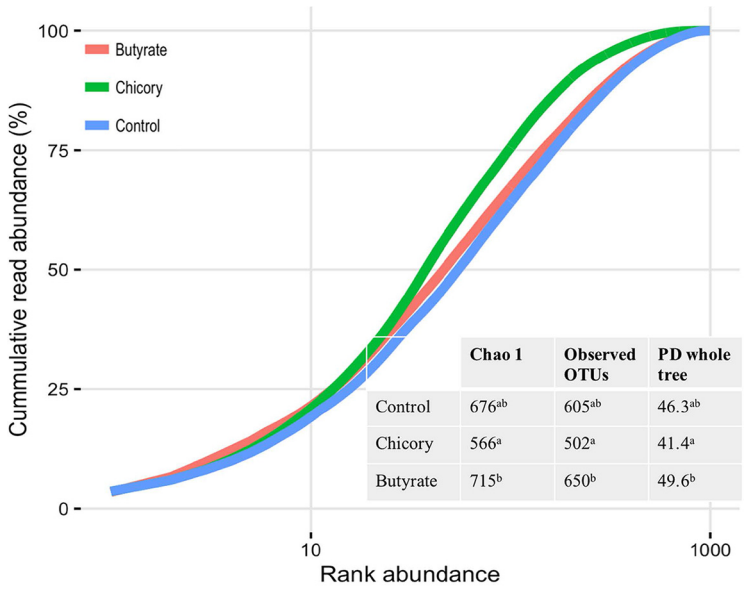

FIG 4 Alpha diversity of fecal samples from the three experimental treatments, using cumulative rank abundance curves and diversity indices (Chao 1, observed OTUs, and PD whole tree). Different letters indicate significant differences among treatments ($P < 0.05$).

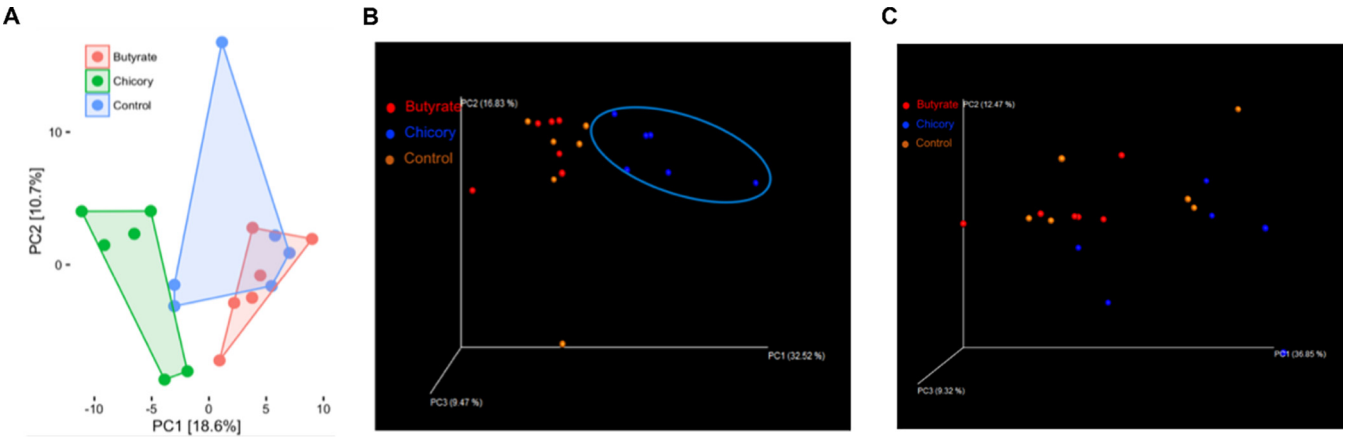

FIG 5 Beta diversity. (A) Principal-component analysis (PCA) based on the square root transformed OTU counts; (B) principal-coordinate analysis (PCoA) based on unweighted UniFrac distances; (C) weighted Unifrac distances of fecal samples from the three experimental treatments (each point represents the composition of fecal microbiota of one pig).

pattern among the different treatments was further confirmed by cumulative rank abundance curves (Fig. 4).

Beta diversity. Principal-component analysis (PCA) and principal-coordinate analysis (PCoA) were used to illustrate beta diversity of the three treatment groups and visualize possible differences among them (Fig. 5). The observed community composition of the chicory group was distinguished from those of the butyrate and control groups, whereas there was an overlap between the control and the butyrate groups (Fig. 5A). Analysis of similarities (ANOSIM) of unweighted UniFrac distances (accounts for the relative abundance of OTUs) indicated that the control, chicory, and butyrate groups were different ($P = 0.010$; $R = 0.260$). The PCoA plot of the unweighted UniFrac distances (only considers their presence or absence) visually confirmed that the chicory group formed a distinct cluster separated from the butyrate and the control groups (Fig. 5B). In contrast, no clear visual separation among the treatments was observed in the PCoA plot of the weighted UniFrac distances (Fig. 5C), despite the ANOSIM of weighted UniFrac distances also showing that the treatments were different ($P = 0.040$; $R = 0.162$).

DISCUSSION

In the current study, we found that a physiologically relevant rate of intracecal butyrate infusion, which did not cause obvious health problems to the pigs, did not decrease skatole levels in feces or plasma. In fact, higher values of skatole were detected, with significant differences from the chicory group. In general, a tendency for increased levels of *p*-cresol and BCFA in the feces of pigs receiving intracecal butyrate infusion was noted. *p*-Cresol, isobutyrate, and isovalerate are formed from tyrosine (23), valine (24), and leucine (24) degradation, respectively. These findings indicate that protein fermentation was somewhat increased in response to intracecal butyrate infusion. This could be the result of stimulation of certain proteolytic bacteria in response to exogenous butyrate. However, the data on microbiota composition are not solid enough to make clear conclusions on this aspect.

The higher proteolytic activity resulting in an increased skatole concentration in the animals receiving exogenous butyrate was unexpected. One explanation could be increased cell apoptosis in either the small intestine or the large intestine due to the butyrate infusion. It is worth noting that the effect of cecal butyrate infusion on intestinal cell proliferation and apoptosis is controversial (25, 26). Further, the *in vivo* effect of butyrate on cell apoptosis and proliferation has been found to be dependent on the dietary lipid source (27). Whether butyrate stimulates (proliferation) or inhibits (apoptosis) cell growth may depend on the availability of other specific energy sources, providing an explanation for the apparent contradictory effects of butyrate on cell

growth. The current results indicate that exogenous butyrate alone does not prevent skatole formation; in fact, a tendency toward the opposite was observed.

Butyrogenic and bifidogenic effects of inulin-type fructans in humans have been reported in various studies (28–30). However, in the present study, fecal SCFA levels, including butyrate, were not affected by the high level of dietary chicory root supplementation. In line with our study, Rideout et al. and Vhile et al. (13, 31) found no impact of feeding diets containing 5% inulin extract, 9% chicory inulin, or 12.2% Jerusalem artichoke on fecal butyrate concentration. Similarly, in humans, no significant changes were observed on fecal SCFA levels following inulin supplementation (32). As already mentioned, intracecal butyrate infusion did not result in an increased concentration of butyrate in feces in the present study. Since up to 99% of the SCFA produced in the hindgut are absorbed rapidly (33), butyrate levels in feces do not accurately reflect its production or concentration in the hindgut. This was also pointed out by Vhile et al. (13).

Microbial degradation of TRP results in the production of skatole, indole, and IPA, with IAA as an intermediate product in skatole production (34). In our study, feeding high levels of chicory roots showed a reduction in fecal skatole concentration, a tendency toward an increased level of IPA, but no effect on fecal indole or IAA concentrations. Similar results were obtained by Knarreborg et al. (34) when feeding entire male pigs with a diet supplemented with 10% sugar beet pulp. These data may indicate that DF fermentation altered the microbial metabolism of TRP toward IPA production at the expense of skatole. However, due to the relatively low concentration of IPA measured, IPA is not considered to totally account for the reducing effect of DF on skatole production. It has been suggested from *in vitro* studies that the skatole-reducing effect of sugar beet pulp and FOS is through shifting the microbial metabolism of TRP to indole production at the expense of skatole (35). Vhile et al. (13) proposed a similar mechanism, although they found, as in the current study, that the decreased skatole production was not accompanied by increased indole levels.

Until now, skatole production has been confirmed in only four bacterial species belonging to only two genera, i.e., *Clostridium* (36) and *Olsenella* (3). Unlike *C. scatologenes* and *C. drakei*, which have been isolated from soil and sediment, respectively, and *O. uli*, which has been isolated from the human oral cavity, *O. scatoligenes* is the only reported skatole producer isolated from the pig gut (3). Adding Jerusalem artichoke to feed reduced the growth of *C. perfringens* (13), which the authors postulated could potentially be used as an indicator of the *Clostridium* genus in general. Thus, they hypothesized that adding high levels of fermentable DF results in reduced growth of the skatole-producing bacteria *Clostridium* in the hindgut of pigs and hence reduced skatole synthesis (13). In our study, the skatole-reducing effect of chicory roots did not coincide with a reduced number of *O. scatoligenes* organisms, which was also reported by Li et al. (37). On the contrary, an increased number of *O. scatoligenes* by chicory roots was detected, which is in line with the studies of Mao et al. and Haenen et al. (38, 39), who reported increased proliferation of *Olsenella* genus by DF. One possible explanation for the observed proliferation of *O. scatoligenes* in the chicory group could be that skatole production, which is not an essential metabolic function of *O. scatoligenes*, is suspended during the exponential growth phase when there is sufficient supply of fermentable carbohydrates. At the same time, available amino acids, including TRP, are incorporated into bacterial biomass instead of being used for skatole production, leading to bacteria growth. On the other hand, in the control and the butyrate groups, with fewer available carbohydrates, *O. scatoligenes* starts drawing its energy for maintenance from protein fermentation, which among other metabolites, results in a higher production of skatole as an end product, while bacterial growth is hindered. Thus, it can be inferred that the reducing effect of chicory roots on skatole production is not by inhibiting the growth of this skatole-producing bacterium in the pig hindgut.

Further, the number of cultivable total anaerobes was significantly higher in the feces of the chicory group (10.11 log CFU/g) than of the other two groups (9.75 to 9.87 log CFU/g) ($P = 0.01$) (data not shown). Thus, the mode of action of the reducing effect

of chicory on skatole production is most likely through increased microbial activity, with a corresponding high incorporation of amino acids into bacterial biomass and thereby suppressed conversion of tryptophan into skatole, as indicated in the literature (7, 9).

In line with data from the literature, the two most abundant phyla in our study, representing in average approximately 76% of the total sequences, were *Firmicutes* and *Bacteroidetes* regardless of treatment. *Cyanobacteria*, although they are photosynthetic organisms, were evident in pig feces. The numerically higher recovered *Cyanobacteria* in the chicory group might be of feed origin. Surprisingly, in the present study, *Treponema* was the most abundant genus, which is in contrast to previous reports often listing *Prevotella* as the dominant genus in the pig gut (40–42). *Treponema* has also been reported as a relatively abundant genus, though (42). The growth of *Treponema* spp. can be enhanced by carbohydrates (43). Niu et al. (44) also found *Treponema* as one of the most abundant genera, and its abundance positively correlated with apparent crude fiber and acid detergent fiber digestibility. In the current study, we did not see a higher abundance in relation to increased DF (chicory group).

At the phylum level, there were no significant differences among treatments in the proportion of taxonomic groups of the fecal microbiota. However, there were statistical differences when analyzed at the genus level, yet they were limited to only a few genera. The abundances of *Bacteroides*, *Collinsella*, *Roseburia*, *Paludibacter*, *Enterococcus*, and *Coprococcus* were significantly influenced by treatment. Cho et al. (45) found that the production of skatole and *p*-cresol was positively correlated with *Bacteroides*, whereas no such relationship was observed in our study. Chicory roots and butyrate inclusion resulted in a decreased relative abundance of *Bacteroides* with a concomitant increased skatole concentration. We observed that the butyrate group contained the highest proportion of unclassified genera within *Clostridiales* (1.3%) and *Bacteroidales* (3.6%), which contain members of proteolytic species that are capable of metabolizing aromatic amino acids (e.g., tryptophan and tyrosine) (46). It may be speculated that the enrichment of these proteolytic species was responsible for the observed skatole and *p*-cresol increases after butyrate infusion. A more thorough analysis at the species level would, however, be needed in order to prove this statement.

With regard to the butyrate-producing bacteria, *Acidaminococcus* and *Megasphaera*, within clostridial cluster IX, and *Coprococcus*, *Eubacterium*, and *Roseburia*, within clostridial cluster XIVa, are dominant in the pig gut microbiota (11). *M. elsdenii* is a well-known lactate fermenter (to butyrate and propionate), and its abundance has been found to increase when pigs are fed a diet added chicory roots (10, 12). In our study, *Megasphaera* was numerically higher, while *Roseburia* was significantly lower, when chicory roots were added to the diet. Therefore, chicory roots may selectively promote the growth of specific butyrate-producing bacteria such as *M. elsdenii* in pig hindgut. However, the skatole-reducing effect of chicory roots was not associated with a general increase in the population of butyrogenic bacteria.

Alpha diversity indices and rarefaction analysis showed lower bacterial species richness in the fecal microbiota of pigs receiving chicory roots than in those receiving butyrate infusion. On the other hand, higher richness has been found for pigs fed 1.5% inulin than for control pigs using denaturing gradient gel electrophoresis (47). Beta diversity analysis revealed an influence of treatment on fecal microbiota composition. Both PCA and unweighted UniFrac PCoA plots showed that the chicory group formed a distinct cluster, whereas the control and butyrate groups, which were both fed a standard Danish grower diet, clustered more closely. However, a clear separation was not observed in the weighted UniFrac PCoA plots. Weighted UniFrac accounts for the relative abundance of OTUs, whereas unweighted UniFrac only considers their presence or absence (48). Unweighted UniFrac is most informative when communities differ primarily by the members present, in part because abundance information can obscure significant patterns of variation in which taxa are present or not (49). The current data indicated that chicory root supplementation had an effect on the microbiota by affecting the presence or absence of OTUs rather than modulating their relative abundances.

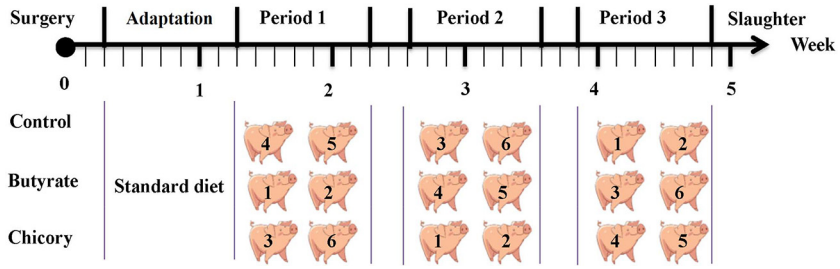

FIG 6 Distribution of pigs in diets and periods. Six animals were surgically fitted with a urine catheter in the cecum. The control group received a standard diet and cecal infusion of NaCl, the butyrate group received a standard diet and cecal infusion of butyrate, and the chicory group received a chicory diet and cecal infusion of NaCl. The duration of each experimental period was 7 days, in which pigs were housed in metabolic cages, followed by a 2-day period in which pigs were moved to individual pens and fed a standard Danish diet.

In conclusion, the data of this study indicated that the skatole-reducing effect of chicory roots is neither via increased butyrate nor via suppression of skatole-producing bacteria in the pig hindgut. An increased microbial activity due to high levels of fermentable fiber, with a corresponding high incorporation of amino acids into bacterial biomass, resulting in reduced conversion of tryptophan into skatole, is probably the mode of action.

MATERIALS AND METHODS

Ethics statement. Animal experimental procedures were carried out in accordance with the Danish Ministry of Justice, law no. 253 of March 2013 concerning experiments with animals and care of experimental animals, and license (no. 2013−15−2934−00818) issued by the Danish Animal Experiments Inspectorate, Ministry of Food, Agriculture and Fisheries, Danish Veterinary and Food Administration.

Animals and experimental design. A total of six crossbred [Duroc × (Danish Landrace × Yorkshire)] entire male pigs with an initial body weight of 41.1 ± 1.8 kg and at an age of ~3 months were used. The experiment followed a Latin square design with 3 treatments, 3 periods, and 6 animals, resulting in 2 animals per treatment per period, i.e., a total of 6 animals per treatment (Fig. 6). All animals were surgically fitted with a urine catheter into the cecum as described by Jørgensen et al. (50). All pigs received cefuroxime the day of operation and streptocillin for the first 3 days after the operation. After a 1-week recovery period in individual pens (1.65 by 1.50 m), the pigs were moved to individual stainless steel metabolic cages. The duration of each experimental period was 7 days, in which the animals were housed in the metabolic cages and received the experimental treatments. The weight of feces produced by each pig was recorded daily. On day 6 of each period, pigs were fitted with a permanent jugular vein catheter. Further, on the same day, a fecal sample from each pig was taken directly from the rectum for quantitative real-time PCR (qPCR), 16S rRNA gene sequencing, and SCFA, dry matter, and phenolic and indolic compound determination. On day 7 of each period, blood samples from the jugular vein were taken 30 min before feeding and at 2, 4, and 6 h after feeding and analyzed for indolic compound concentration. The collected samples were immediately frozen and stored at −20°C until analyzed. Each experimental period was followed by a 2-day period in which the pigs were housed in individual pens and fed with a standard Danish grower diet.

Diets and infusion. Two diets were formulated: a standard Danish grower diet based on wheat, barley, and soybean meal, and a diet containing 25% chicory root (Table 3). The pigs were fed daily at 07:00 and 14:30. The daily amount of feed offered to the animals was adjusted weekly to 4% of their body weight (BW). One hour after each meal, leftover feed (if any) was removed and recorded. The study included three experimental groups: the control group received the standard diet and a cecal infusion (25 ml/h) of 0.9% NaCl, the chicory group received the chicory diet and a cecal infusion (25 ml/h) of 0.9% NaCl, and the butyrate group received the standard diet and a cecal infusion (25 ml/h) of a 200 mM butyric acid solution, corresponding to a daily infusion of 132 mmol of butyrate, for 7 days in each period. The amount of butyrate infused was based on the amount estimated to be produced in the hindguts of growers in a study by Anguita et al. (33) and in a previous infusion study (50). To obtain a physiological pH of 5.1, 80% of the carboxylic groups of the butyric acid were neutralized with 120 ml/liter of a neutralizing solution consisting of 0.13 mol of $CaCO_3$/liter, 0.65 mol of NaCl/liter, and 0.50 mol of KOH/liter (51). To obtain an ionic strength of the butyric acid solution similar to 0.9% NaCl, the butyric acid solution was prepared by dissolving 17 ml of butyric acid, 120 ml of neutralizing solution, and 500 ml of 0.9% NaCl in 363 ml of H_2O.

Analytical methods. The concentration of SCFA was determined by gas chromatography as outlined by Canibe et al. (52). The concentration of phenolic and indolic compounds in feces and indolic compounds in plasma was analyzed by high-performance liquid chromatography (HPLC) as described by

TABLE 3 Ingredients and the calculated composition of the experimental diets

Item measured	Value for group(s)	
	Control and butyrate	Chicory
Ingredient composition (%)		
Wheat	55.97	28.83
Barley	20.00	20.00
Dehulled toasted soybean meal	9.93	12.00
Dehulled sunflower cake	8.00	8.00
Dried milled chicory root[a]		25.0
Soybean oil	1.70	1.40
Sugar beet molasses	1.50	2.00
Calcium carbonate	1.42	1.33
Monocalcium phosphate	0.36	0.35
Sodium chloride	0.49	0.48
L-Lysine-HCl, 98%	0.32	0.29
DL-Methionine, 98%	0.03	0.04
Threonine, 98%	0.08	0.08
Vitamin and mineral premix[b]	0.20	0.20
Calculated composition (g/kg of DM)		
DM	862	886
Fat	37	30
Crude protein	151	150
Ash	50	59
Calcium	7.2	8.4
Phosphorus	4.5	4.5

[a]The content of fructan in the dried milled chicory root was 65.2%.

[b]Supplied per kilogram of diet: 4,200 IU of vitamin A, 420 IU of vitamin D_3, 69.2 mg of vitamin E, 2.1 mg of vitamin B_1, 2.1 mg of vitamin B_2, 3.2 mg of vitamin B_6, 20 μg of vitamin B_{12}, 63.0 mg of DL-alpha-tocopherol, 69.2 mg of DL-alpha-tocopherol acetate, 10.5 mg of Ca-D-pantothenic acid, 21 mg of niacin, 20 μg of biotin, 2.1 mg of vitamin K_3, 84 mg of Fe [Fe(II) sulfate], 15 mg of Cu [Cu(II) sulfate], and 42 mg of Mn [Mn(II) oxide].

Knarreborg et al. (34). The plasma skatole detection level was 0.3 ng/ml. The DM content of the fecal samples was measured by freeze-drying.

Microbial genomic DNA extraction. Microbial genomic DNA (gDNA) from approximately 200 mg of fresh feces was extracted using the QIAamp Fast DNA stool minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions for pathogen detection. The DNA quality was measured by a NanoDrop 1000 spectrophotomer (Thermo Scientific, Waltham, MA), and the DNA concentration in each sample was quantified with the Qubit fluorometer 3.0 (Life Technologies, Grand Island, NY). gDNA was stored at −20°C prior to qPCR and 16S rRNA gene sequence analysis.

Quantitative real-time PCR. Quantitative real-time PCR for enumeration of *O. scatoligenes* in feces was performed based on a previously developed TaqMan-MGB qPCR assay (37), using a ViiA 7 real-time PCR system (Applied Biosystems, Foster City, CA) associated with ViiA 7 RUO software version 1.2.1 (Life Technologies). The species-specific primer and probe pair used were as follows: OscF, 5′-CTTACCAGGG CTTGACATCTTGG-3′ (positions 949 to 971); OscR, 5′-ACGACACGAGCTGACGACAG-3′ (positions 1043 to 1024) (obtained from DNA Technology A/S, Denmark); and OscPR, 5′-6-carboxyfluorescein (FAM)-ACCT GTCTTGGCTCCT-MGB-NFQ-3′ (positions 1014 to 999) (purchased from Applied Biosystems, Life Technologies, UK). Amplifications were carried out in a total volume of 10 μl consisting of 5.0 μl of TaqMan Universal master mix (Applied Biosystems), 300 nM each primer, 200 nM TaqMan-MGB probe, and 2 μl of gDNA template. Approximately 10 ng of DNA as measured by the Qubit 2.0 fluorometer (Invitrogen) was used. The qPCR program was as follows: hold for 2 min at 50°C, followed by 10 min at 95°C and then 40 cycles of 15 s at 95°C and 1 min at 60°C. Each qPCR run included a negative control, a gDNA standard consisting of *O. scatoligenes* SK9K4^T gDNA with known concentrations, and a reference standard consisting of a serial dilution of DNA from 200 mg of feces spiked with 1.5×10^{10} cells/g of SK9K4^T. All reactions were conducted in triplicate. All qPCR data were quantified against the reference standard. Results were reported as log *O. scatoligenes* cells per gram of feces (wet weight).

16S rRNA gene sequencing. The V3-V4 regions of the 16S rRNA gene were amplified according to the 16S metagenomic sequencing library preparation protocol (Illumina, San Diego, CA) as described previously (53), with modifications. Briefly, the first PCR, with the bacterial primers Bac341F (5′-CCTAC GGGNGGCWGCAG-3′; positions 341 to 357) and bac805R (5′-GACTACHVGGGTATCTAATCC-3′; positions 785 to 805) (54), was performed using a 25-μl assay mixture containing 12.5 μl of 2 × KAPA HiFi HotStart ReadyMix (Kapa Biosystems Inc., Wilmington, MA), 0.5 μl of forward primer (0.1 μM), 0.5 μl of reverse primer (0.1 μM), 1.0 μl of bovine serum albumin (BSA), 8.5 μl of H_2O, and 2.0 μl of DNA template. The program was 95°C for 0.5 min and 20 cycles of 55°C for 0.5 min, 72°C for 0.5 min, and 72°C for 5 min. The second PCR, amplified with the overhang adapters (5′-TCGTCGGCAGCGTCAGATGTGTAT AAGAGACAG-locus-specific sequence-3′ and 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-locus-specific sequence-3′), was performed following the same procedures except that only 10 amplification cycles were conducted. The third PCR amplification, with added Nextera index primers

(Illumina Nextera XT v2; Illumina), was performed following the same procedures except that only eight amplification cycles were conducted. The PCR products from each reaction were purified using magnetic bead technology (Agencourt AMPure XP, Beckman Coulter Inc., Brea, CA) according to the manufacturer's instructions. The size and the quality of all amplicons were checked using 1% agarose gel electrophoresis. The final DNA concentration of all libraries was quantified fluorometrically by Qubit 2.0 using a high-sensitivity assay kit (Invitrogen) and then pooled in equimolar concentrations and diluted to a final DNA concentration of 4 nM. The sample pool was sequenced with an Illumina MiSeq (Microarray Facility, VU University Medical Center [VUmc], Amsterdam, the Netherlands) following a 2 × 300-bp paired-end protocol at the Center for Geomicrobiology, Department of Bioscience, Aarhus University, Aarhus, Denmark.

Bioinformatic analysis. Raw Illumina FASTQ sequences were initially demultiplexed using Miseq Reprotrer V2.0 and then analyzed using QIIME (Quantitative Insights Into Microbial Ecology) software package version 1.9.1 (55). Paired-end reads were first merged by multiple_join_paired_ends.py and followed by extract_barcodes.py to remove forward and reverse primer sequences. In QIIME, multiple_split_libraries_fastq.py was used to demultiplex and quality filter, using a minimum quality score of 25. The pick_open_reference_otus.py script in QIIME was used to pick OTUs and assign taxonomy using the uclust method (56) with the Greengenes references (13_8 release) database (57) and a 97% similarity threshold. For *Olsenella* taxonomic classification, the final sequences generated from QIIME split_libraries were analyzed using the Ribosomal Database Project (RDP) classifier (https://pyro.cme.msu.edu/classifier/form.spr) (58) with an 80% confidence threshold. OTUs representing fewer than 0.005% of all sequences were discarded using filter_otus_from_otu_table.py script (59). Alpha diversity (estimated by Phylogenetic Diversity [PD] Whole Tree, Chao 1, and Observed Species indices) and beta diversity (estimated through UniFrac distances) were calculated using the QIIME workflow core_diversity_analysis.py, with a sampling depth of 39,346 (48). R version 3.2.5 (R Development Core Team 2014) with the R package ampvis v.1.25.0 (https://github.com/MadsAlbertsen/ampvis) (60) was further used for the analysis of the sequencing data. Principal-component analysis (PCA) was conducted using square root transformed OTU counts in Vegan (http://CRAN.R-project.org/package=vegan). OTUs in differential abundance between treatments were identified by DESeq2 (61) using test = "wald" and fitType = "parametric."

Statistical analysis. The model used to analyze the variables from feces was a mixed model including treatment, period, and their interaction as fixed effects. The repeated statement was used to account for correlation among samples taken from the same pig. Compound symmetry was used as the covariance structure. The model used to analyze the plasma data included treatment, period, time of sampling, and their interactions. The repeated statement was included to account for the various samples taken from the same pig at various time points, and the random pig effect was included to account for samples taken from the same pig in the three periods. Autoregressive was used as the covariance structure. The statistical analyses were performed with SAS (version 9.4; SAS Institute Inc., Cary, NC). Differences between means were compared by Tukey's least significant difference. Significance was declared at a P value of <0.05.

Data availability. The raw reads used in this study were deposited in the Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra) under accession number SRP089699.

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